

MicroRNA-146a, a therapeutic target and biomarker for peripartum cardiomyopathy

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Supplemental tables

Supplemental Table 1: Cardiac function in mice systemically injected with 16K-Ad and Control-Ad

	Control-Ad, baseline	16K-Ad, baseline	Control-Ad, 14 days	16K-Ad, 14 days
FS (%)	43±7	44±6	47±5	32±7*†
LVEDD (mm)	3.2±0.3	3.3±0.2	3.1±0.3	3.5±0.1
LVESD (mm)	1.8±0.3	1.8±0.1	1.7±0.2	2.3±0.2‡§

Baseline: prior injection with respective adenovirus (i.v. via the tail vein). Fractional shortening (FS), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and heart rate were determined by echocardiography, heart rate was similar in both groups at baseline or 14 days after adenovirus injection (data not shown). * $P<0.05$, § $P<0.01$ vs. corresponding baseline group; † $P<0.05$, ‡ $P<0.01$ 16K-Ad vs. Control-Ad 14 d after injection, $n=5$ per group, all male mice 12 weeks of age.

Supplemental Table 2: Follow up of patients with PPCM treated with standard therapy for heart failure and bromocriptine

	PPCM baseline ($n=7$)	Follow up with BR treatment ($n=7$)	p value
LVEF (%)	21±13 (range 9-45)	52±16 (range 30-76)	0.0044**
LVEDD (mm)	60±7 (range 51-67)	55±7 (range 47-66)	0.1376

Baseline: at time of initial diagnosis prior to bromocriptine (BR) treatment (6.5±5 months). Left ventricular ejection fraction (LVEF) and left ventricular end-diastolic diameter (LVEDD) were determined by echocardiography. ** Comparing the change from baseline to 5 months in bromocriptine-treated PPCM patients ($P<0.01$).

Supplementary Data

Treatment with recombinant Neuregulin-1 attenuates PPCM in CKO mice

To assess the importance of impaired ErbB signaling for the development of PPCM, CKO mice were injected with recombinant NRG1 spanning 2 pregnancies and nursing periods. Dose-response analysis indicated that 1.25 µg i.p. was sufficient to activate ErbB signaling in the heart as indicated by activation of ERK1/2 (Figure S4N). This dose, daily applied for two subsequent nursing periods moderately attenuate cardiac dysfunction compared to NaCl treated CKO mice (Figure 4N).

Supplemental methods

RNA extraction, miRNA, pri-miR, mRNA expression analysis by TaqMan MicroRNA Assay and Quantitative Real-time PCR Analysis

Total RNAs were extracted with the miRNeasy kit (Qiagen). Taqman methods were used to assess miRNA expression. Briefly, 10 ng RNA was reverse transcribed to cDNA with the Taqman microRNA Reverse Transcription kit and the Taqman microRNA assay stem loop primers (Applied Biosystems). Resulting cDNAs were used for quantitative real-time PCR using Taqman microRNA assay and Taqman universal PCR master mix reagents (Applied Biosystems). Thermal cycling was performed on an Applied Biosystem 7900 HT detection system (Applied Biosystems). The relative miRNA levels were normalized to two internal controls, RNU-44 or RNU-48 for human cells and sno-202 for rat cells. The relative miRNA levels were normalized to two internal controls, sno-135 and sno-202 (Applied Biosystems) for mouse tissues. For the plasma and exosomes, the relative miRNA levels were normalized to two spikes-in miRNAs: cel-miR-39 and cel-miR-238 (Applied Biosystems).

For mRNA expression analysis, RNAs were extracted with the miRNeasy kit (Qiagen) according to the manufacturer's protocol. cDNA synthesis was performed with 1 µg total RNA and the iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's instructions. Resulting cDNAs (20 ng) were used for quantitative real-time PCR using the SYBR green method (Roche Applied Sciences). Thermal cycling was performed on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). For all reactions, no-template controls were run, and random RNA preparations were also subjected to sham

reverse transcription to check for the absence of genomic DNA amplification. Quantitative real-time PCR was performed with SYBR green method (Bioline and Thermo Fisher Scientific). Thermal cycling was performed on an Applied Biosystem 7900 HT detection system and a Stratagene MX3005P multiplex QPCR system (Applied Biosystems and Stratagene). The relative transcript level of each gene was normalized to the housekeeping genes cyclophilin-A (PPIA), beta-2 microglobulin (B2M) and/or GAPDH. Primers were designed using Primer Express software and selected to span exon-exon junctions to avoid detection of genomic DNA (primer sequences are provided below).

For pri-miR expression analysis, cDNA was produced as described above. Resulting cDNAs (20 ng) were used for quantitative real-time PCR using Taqman pri-miR assay (Applied Sciences).

Northern blot analysis

Total RNA from pre-miR-146a and pre-miR-ctrl transfected cardiomyocytes was isolated using Trizol (Life Technologies) after 5 days. RNA (10 µg) was electrophoresed in an 16% denaturing polyacrylamide gel and transferred by electroblotting onto Hybond-N membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization and washing were carried out at 37° C according to standard protocols. Membranes were probed with a ³²P-labeled 22-nt miR-146a LNA oligonucleotide with the following sequence 5'-AACCCATGGAATTCAGTTCTCA-3' (Exiqon, Vedbaek, Denmark) and were subsequently visualized by autoradiography. Serial dilutions (1 to 0.01 µmol/l) of the synthetic miR-146a RNA (5'-UGAGAACUGAAUCCAUGGGUU-3', BioSprings, Frankfurt, Germany) were subjected to the gel. Endogenous U6 snRNA served both as an internal size standard and a loading control (U6sn LNA probe: 5'-CACGAATTTGCGTGTCATCCTT -3', Exiqon).

Construction of S-miR-146a-IEW lentiviral vector

To construct the lentiviral vector S-miR146a-IEW containing the miR-146a, the plasmid SIEW was digested with *Bam*HI followed by a DNA polymerase I fill-in reaction and gel purification. The miR-146a cassette was excised from pEP-miR-146a plasmid with *Nhe*I and *Bam*HI, and the cohesive ends were filled in using Klenow. The blunt-end fragment was ligated with the blunted *Bam*HI vector fragment placing miR-146a downstream of the SFFV promoter (SmiR-146a-IEW). The preparation of recombinant lentiviral supernatants were performed as described earlier (1).

Transduction of miR-146a and Immunoprecipitation of Human Argonaute 2 Complexes with the RIP kit

Lentiviral supernatants expressing miR-146a and control vector SIEW were used to transduce approximately 1×10^7 NRCM cells with an MOI of ~2. Cells were scraped off 96 h after transduction with either miR-146a or control vector. microRNA:mRNA immunoprecipitation was performed using the Magna RIP kit (Millipore, Billerica, MA, USA) following the manufacturer's protocol. For RISC-IPs of NRCM, 1×10^7 cells were taken for each replicate, washed in PBS before lysis in 100 μ l complete RIP-lysis buffer and incubated with magnetic beads conjugated with anti-Ago2/eIF2C2 antibody (Abcam, Cambridge, UK) control normal mouse IgG (Millipore) and rotated overnight at 10°C. Co-immunoprecipitated RNA, including miRNA:mRNA complexes was subjected to qRT-PCR and miR-qRT-PCR. Expression of mature miRNAs was determined by miR-qRT-PCR using miRNA-specific looped RT-primers and TaqMan probes as recommended by the manufacturer (Applied Biosystems). Normalization was performed using the $2^{-\Delta\Delta CT}$ method relative to U6snRNA.

Microarray Experiment

Microarray experiments have been performed by the GIGA Genomics Facility (Liège, Belgium). After transfection with 50 nM of pre-miR-146a or pre-miR-control for 24 h, total RNA was isolated from HUVECs using RNeasy Mini Kit (Qiagen). Extracted RNA yields were determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific). The purity and quality of extracted RNAs were evaluated using the Experion RNA StdSens Analysis kit (Bio-Rad). High quality RNAs with RNA Quality Indicator (RQI) score greater than 8 were used for microarray experiments. Gene expression profiling was performed using Illumina's multi-sample format Human Ref-8 BeadChip that contains 24,500 transcripts and profiles eight samples simultaneously on a single chip (Illumina Inc.). For each sample, 250 ng of total RNA were labeled using an Illumina TotalPrep RNA Amplification kit (Ambion) according to the manufacturer's instructions. Briefly, double-stranded cDNAs were synthesized using T7-oligo (dT) primers and followed by an in vitro transcription reaction to amplify cRNAs while biotin was incorporated into the synthesized cRNA probe. The cRNA probe was then purified and quantified using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific). Biotinylated cRNA probe was hybridized to the Human Ref-8 BeadChip Array (Illumina). Labeled cRNAs (750 ng) were used for hybridization to each array. The hybridization, washing, and scanning were performed according to the manufacturer's instructions. The arrays were scanned using a BeadArray Reader (Illumina).

Microarray Data Analysis

The microarray images were registered and extracted automatically during the scan according to the manufacturer's default settings. Raw microarray intensity data were analyzed with the GenomeStudio software normalized using the quantile normalization method according to the manufacturer's recommendation. Differential analysis were performed for each group and data were filtered on Differential Score higher than 30 or lower than – 30. The probes were considered as expressed by filtering data on Detection *P*-value lower than 0.01. Data were further analyzed using Ingenuity Pathway software. Microarray data have been deposited in Gene Expression Omnibus (GEO) database (accession number GSE43016).

Sylamer analysis

Sylamer analysis was used to analyze miRNA binding site differences in 3'UTR regions of human genes (2). The analysis was performed on the data generated from the microarray data analysis (see above). The mRNA gene list was ranked from most to the least down-regulated genes and uploaded into SylArray (www.ebi.ac.uk/enright-srv/sylarray).

Preparation of cell extracts

Cells were washed twice with cold PBS and scraped into lysis buffer [25 mM HEPES (pH 7.9), 150 mM NaCl, 0.5 % Triton, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride] on ice. Insoluble cell debris was removed by centrifugation at 10,000 g for 15 min. Aliquots of protein-containing supernatant were stored at -80 °C. Protein concentrations were determined by the Bradford method, with the Bio-Rad protein assay reagent (Bio-Rad Laboratories).

Western Blot Analysis

Soluble cell lysate (30 µg) was resolved by SDS-PAGE (12 %) and transferred to a polyvinylidene fluoride membrane (Milipore Corp.). Blots were blocked overnight with 8 % milk in Tris-buffered saline with 0.1 % Tween 20 and probed for 1 h with primary antibodies: anti-p65 (sc-109, Santa Cruz Biotechnology), anti-beta-actin (ab8226, Abcam), anti-NRAS (sc-31, sc-209, Santa Cruz), anti-ERBB4 (4795S, Cell Signaling), anti-ERK (9102, Cell Signaling), anti-phospho-ERK (9101S, Cell Signaling) and anti-beta-tubulin (ab6046, Abcam). After three washes with Tris-buffered saline containing 0.1 % Tween 20, antigen-antibody complexes were detected with peroxidase-conjugated secondary antibody and an

enhanced fluoro-chemiluminescent system (ECL; Pierce Biotechnology). Ponceau red staining was used to control sample loading and for normalization of animal blots. Quantifications were performed using Image J software and are presented in bar graph normalized to the level of corresponding loading control (beta-tubulin, beat-actin or Ponceau red).

NF- κ B Binding Activity

The p65 DNA-binding activity of NF- κ B was quantified by ELISA (EZ Transcription Factor kit NF- κ B p65 (Pierce)) according to manufacturer's instructions on total protein extracts. NF- κ B binding to the target oligonucleotide was detected by incubation with primary antibody, specific for the activated form of p65, visualized by anti-IgG horseradish peroxidase conjugate and developing solution. Chemiluminescence was quantified with a luminometer.

Luciferase Assay (3'UTR reporter assays)

HEK293T cells were transfected with 30 pmol of pre-miR-Ctrl or pre-miR-146a with 0.6 μ l of DharmaFECT-4 (Dharmacon Research Inc). The next day, cells were transfected with 25 ng of the psiCHECK2 vector (Promega) expressing the 3'UTR of human *NRAS* mRNA (Imagen) or the mutated 3'UTR of human *NRAS* mRNA (QuickChange II Site-Directed Mutagenesis Kit, Agilent) with JET-PEI (Polyplus transfection) following manufacturer's instructions. Wild-type region of 3'UTR human *NRAS* mRNA binding seed sequence of miR-146a: 5'-AGUUCUCA-3'. Mutated region of 3'UTR human *NRAS* mRNA binding seed sequence of miR-146a: 5'-UGUUCAUG-3'. Forty-eight hours later, luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was normalized to Firefly luciferase.

Luciferase Assay (promoter reporter assays).

The 600bp sequences containing the two NF- κ B binding sites wildtype or mutated (3) were synthesized (Genescript) and cloned into the pGL3 reporter vector (Promega) in the *HindIII* et *XhoI* sites, located upstream of the Firefly Luciferase coding sequence. HUVEC (80,000/well in 12-well plates) were transfected with 1 μ g of the vector and 0.025 μ g of the control vector pRenilla-Luc using XTreme Gene HP (Roche). After 24 h, cells were treated with 16K-PRL (50 nM). Forty-eight hours later, luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase.

Cell proliferation.

Control and NRAS siRNA-transfected cells or pre-miR/anti-miR-transfected cells were plated in 96-well culture plates at a density of 10,000 cells per well in 100 µl of SFM (Lonza) supplemented with 5 ng/ml bFGF or EGM-2 (Lonza) and 0.5 % serum, respectively and incubated for 72 h. When indicated, the cells were treated with bFGF (10 ng/ml), with or without 16K PRL (50 nM). Proliferation was analyzed 24 h later by measuring BrdU incorporation using the Cell Proliferation ELISA, BrdU (Colorimetric) (Roche).

Caspase-3 activity assay

Control and NRAS siRNA-transfected cells or pre-miR/anti-miR-transfected cells were plated in 24-well culture plates at a density of 20,000 cells per well in 500 µl of SFM (Lonza) supplemented with 5 ng/ml bFGF or EGM-2 (Lonza) and 0.5 % serum, respectively. Caspase-3 activity was measured 48 h post-transfection with the CaspACE Assay System Fluorimetric (Promega Corp., Madison, WI) according to the manufacturer's instructions.

Flow cytometry

Transfected cells or pre-miR/anti-miR-transfected cells were plated in 6-well culture plates at a density of 100,000 cells per well in 500 µl of SFM (Lonza) supplemented with 5 ng/ml bFGF or EGM-2 (Lonza) and 0.5 % serum, respectively. Cells were stained with Annexin-V FITC and propidium iodide (PI) with FITC Annexin-V Apoptosis detection kit II (BD Pharmingen) according to the manufacturer's instructions.

DNA Fragmentation ELISA

Control and NRAS siRNA-transfected cells or pre-miR/anti-miR-transfected cells were plated in 24-well culture plates at a density of 100,000 cells per well in 500 µl of SFM (Lonza) supplemented with 5 ng/ml bFGF or EGM-2 (Lonza) and 0.5 % serum, respectively and incubated for 72 h. DNA fragmentation was measured with the Cell Death^{plus} Detection ELISA kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Scratch wound migration assay

Pre-miR/anti-miR-transfected cells were plated in 48-well culture plates at a density of 50000 cells per well in 350 µl of EBM-2 (Lonza) and 0.5 % serum and incubated for 24 h to

reach confluence. Using a tip, a wound was made in the monolayer (at time 0). The cells were then washed with PBS and incubated with EBM-2 medium containing 10 ng/ml recombinant bFGF (Promega Corp.) and 50 ng/ml recombinant VEGFa (RELIA Tech GmbH) for 16 h. The distance between the two sides of the wound was measured with a graduated ocular lens coupled with an Olympus CKX41 microscope (Olympus). The distance between the two sides of the wound after 16 h of migration was subtracted from the distance at time 0 and represented on a graph.

Capillary tube network formation on a Matrigel matrix (tubulogenesis assay)

Pre-miR/anti-miR-transfected cells were plated in 96-well culture plates pre-coated with 50 μ l Matrigel per well (BD Biosciences) at a density of 10000 cells per well in 100 μ l of EGM-2 (Lonza). The cells were incubated for 16 h. Pictures were taken with an Olympus fluorescence microscope (Olympus). Quantitative analysis of network structure was performed with ImageJ software (<http://rsbweb.nih.gov/ij/>) by counting the number of intersections in the network and measuring the total length of the structures.

Exosome purification, labeling and analysis

Exosomes were purified from conditioned cell culture media as previously described (4). Briefly, medium was collected and centrifuged at 300 g for 15 min and then again at 12,000 g for 35 min. Supernatants were then passed through a 0.22 μ m filter (Millipore) and ultracentrifuged at 110,000 g for 70 min at 4 °C. The pellets were washed with PBS and after a second ultracentrifugation at 110,000 g for 70 min at 4 °C, pellets of exosomes were resuspended in PBS. Exosomes were labeled and analyzed by flow cytometry as previously described (5). Briefly, exosome preparations were incubated with 4-mm-diameter aldehyde/sulfate latex beads (Invitrogen) and resuspended in PBS containing 2% FCS. Exosome-coated beads were incubated with anti-CD63-FITC (Pharmingen), followed by incubation with FITC-conjugated secondary antibody and analyzed on an FACS analyzer (Beckman Coulter). For the fusion experiments, exosomes were stained using the PKH67 Green Fluorescent Cell Linker Mini Kit according to manufacturer's instructions (Sigma-Aldrich). For the dynamic light scattering, the exosomes were suspended in PBS and analyses were performed with a Zetasizer Nano ZS (Malvern Instruments Ltd). Intensity, volume and distribution data for each sample were collected on a continuous basis for 4 min in sets of three.

NRCM were transfected with anti-miR-146a or anti-miR control (100 nM, Ambion) using Lipofectamine (Invitrogen). After transfection for 24 h, cells were washed, treated with exosomes loaded with miR-146a or control exosomes (40ug/ml) and kept for an additional 4 days in serum-free medium. Cells were harvested or cell metabolism was determined by MTS assay (Promega) according to manufacturer's instruction. Cell culture experiments were performed from 3 independent cell isolations

Dynamic light scattering

Exosomes were suspended in PBS and dynamic light-scattering measurements were performed with a Zetasizer Nano ZS (Malvern Instruments Ltd). Intensity, volume and distribution data for each sample were collected on a continuous basis for 4 min in sets of three.

Immunohistochemistry of exosomes in vivo

Fifty µg of exosomes or control solution were injected directly into the heart at three different injection points in adult female WT mice ($n=2$). The animals were euthanized 24 h post-injection. The hearts were extracted, fixed in 2 % paraformaldehyde (PFA), washed in PBS and then incubated in 15 % and 30 % sucrose. Fixed hearts were cryo-sectioned and stained with anti-human CD63 (Abcam ab134045) antibody, sarcomeric α -Actinin (A7811, SIGMA) and DAPI Hoechst 33258 (SIGMA). Images were taken by fluorescence microscopy with Axiovision 4.6 software (Carl Zeiss).

In situ hybridization and immunohistochemistry

On LV cryosections, H&E, isolectin B4 (Vector), wheat germ agglutinin (WGA, Vector), CD31 (BD Pharmingen, 555289) and Hoechst 33258 stainings were performed as described (6). In situ hybridization for miR-146a was performed using a 5' fluorescein labeled 19mer antisense oligonucleotide (Ribotask) containing locked nucleic acid and 2'OME RNA moieties (FAM-AacCcaTggAauTcaGuuCucA, capitals indicate LNA, lower case indicates 2'OME RNA). The hybridizations were done on 6-µm cryosection according to protocol previously described (7) with a hybridization temperature of 60°C. As control, a mutated miR-146a probe (FAM-AcaCacTccAaaTguGuuCauG) was used and gave no specific signal.

Electronic Microscopy analysis

NRCM were treated with exosomes (final concentration 20 µg/ml) dissolved in NaCl or control solution. After 16 h pre-incubation, the NRCMs were washed with PBS and fixed in 2,5 % glutaraldehyde/ 1 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 h at 4°C. Then the cells were washed 3 times in sodium phosphate buffer for 10 min at 4 °C and post-fixed for 30 min with 2 % osmium tetroxide. After dehydration in graded ethanol, samples were embedded in Epon. Ultrathin sections obtained with a Reichert Ultracut S ultramicrotome were contrasted with uranyl acetate and lead citrate. Observations were made with a Jeol JEM-1400 transmission electron microscope at 80 kV.

Sequences of qRT-PCR primers, siRNAs and antagomir

All primers and siRNA and scramble siRNA control were synthesized by IDT-DNA

Gene	Sense primers (5' to 3')	Antisense primers (5' to 3')
hsa p65	AATCCAGTGTGTGAAGAAGCGG	TAGTCCCCACGCTGCTCTTCTA
hsa NRAS	ATTTTTCCTGGCTGTGGTC	CCAACCACCACCAGTTTGTACT
hsa IRAK1	TCTACCAAGCCAGGCCAGAGA	TCCGAGAAGTTGTGGGTGCC
hsa PPIA	CCAACACAAATGGTTCCAGT	CCATGGCCTCCACAATATTCA
hsa B2M	GAGTATGCCTGCCGTGT	AATCCAAATGCGGCATCT
hsa ErbB4	GATTCTCAGTCAGTGTGTGCAGGAAC	TATCTCCAGGTTGCCCATGACAACC
mmu/rno ErbB4	GTGCTATGGACCCTACGTTAGT	TCATTGAAGTTCATGCAGGCAA
mmu NRAS	CTGGCCAAGAGTTACGGAATTC	CATTCGGTACTGGCGTATCTCC
mmu/rno PPIA	ACCGTGTTCTTCGACATCACG	CTGGCACATGAATCCTGGAATA
mmu/rno IRAK1	TCCTCCACCAAGCAGTCAAG	AAAACCACCTCTCCAATCCT
mmu/rno Notch1	CCGTGTAAGAATGCTGGAACG	AGCGACAGATGTATGAAGACTCA
mmu/rno GAPDH	ACCACCATGGAGAAGGCTGG	CTCAGTGTAGCCCAGGATGC
mmu/rno b2m	CATGGCTCGCTCGGTGACC	AATGTGAGGCGGGTGGAAGT

Nom	Brin sens	Brin anti-sens
siRNA p65	GCCCUAUCCCUUUACGUCAdTdT	dTdTCGGGAUAGGGAAUAGCAGU
siRNA control (p65)	UCGACCUCACUCCCAUUGdTdT	dTdTAGCUGGAGUGAAGGGUAAC
siRNA NRAS humain (IDT-DNA)	rArGrC rUrUrA rCrUrG rArUrA rArArC rCrUrA rArUrA rUrUC A	rUrGrA rArUrA rUrUrA rGrGrU rUrUrA rUrCrA rGrUrA rArGrC rUrArG

siRNA NRAS souris (IDT- DNA)	rGrGrC rArGrU rCrArC rArArA rUrUrA rArCrA rUrGrU rUrGC T	rArGrC rArArC rArUrG rUrUrA rArUrU rUrGrU rGrArC rUrGrC rCrGrG
siRNA control (NRAS)	siRNA universal control (IDT-DNA)	

Name	Sequences (5'to3')
Antagomir-control (VBC biotech)	AAGGCAAGCUGACCCUGAAGUU
Antagormir-146a (VBC biotech)	AACCCAUGGAAUUCAGUUCUCA
LNA-miR-146a (hsa-miR- 146a/b-5p, Exiqon)	TGGAATTCAGTTCTC
LNA-miR- control(scramble, Exiqon)	ACGTCTATACGCCA

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